Integration of wild and captive genetic management approaches to support conservation of the endangered Japanese golden eagle

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Abstract:

The loss of biological diversity within species has the potential to significantly reduce resilience in the face of environmental change. Conservation of genetic variation needs to consider all available sources of diversity within a species, and approaches are required to integrate population management across traditionally separate wild and captive population domains. Here we report on a study that utilises different types of genetic analysis at different taxonomic scales and across an *in situ – ex situ* transition to support conservation planning for the Japanese golden eagle, a subspecies in serious regional decline. Mitochondrial DNA sequencing and nuclear DNA profiling are used to investigate subspecies differentiation and diversity in the natural population, revealing relatively high levels of variation in Japan. These results are compared with data from a newly established conservation breeding programme that indicates good representation of wild genetic variation in the captive founders. However, subsequent population viability analysis (PVA) to examine the demographic and genetic future of the captive population demonstrates the severe effects of existing reproductive skews, suggesting that this population is not sustainable without intensive genetic management. Lastly, the use of available molecular tools to validate and reconstruct pedigrees in Japanese golden eagle are evaluated and discussed in the context of captive and wild conservation management. The paper highlights the importance of producing and utilising comparative molecular genetic data across the population management spectrum and the benefits of PVA to support the implementation of integrated conservation plans.

1. Introduction

Despite the best efforts of conservationists, the decline of natural wildlife populations has led to many taxa being on the verge of disappearing from the wild. With notable exceptions, extinction of our most charismatic species has so far been avoided, however we are witnessing huge reductions in both numbers and diversity within species (World Wide Fund for Nature, 2016), with extirpation of populations effectively hollowing-out the genetic variation that enables adaptive responses to local and global environmental change. The issue of intra-specific diversity reduction is now recognized explicitly within the Convention on Biological Diversity (Aichi Target 13 of SCBD, 2010). As well as being of widespread concern to field conservationists, it is the focus of great attention from the *ex situ* conservation community who are responsible for genetic management of threatened species in captivity (Lacy, 1987; Lacy, 2013). Captive genetic management is primarily managed through individual-level pedigree analysis based on studbook records (Balou and Lacy 1995, Ivy and Lacy 2012). However, with incomplete pedigree data in many captive populations limited knowledge of kinship, and a move away from intensive management altogether in some species (Wildt et al. 2012), molecular genetic data are being viewed as the potential solution to validate, contextualise and sometimes simply correct theoretical genetic management estimates (Fienieg and Galbusera 2013). In this regard, the measurement and management of extant genetic diversity provides a common and potentially unifying theme to wildlife conservation, from extensive population management to intensive individual level husbandry.

The synthesis of conservation strategies for a single species across different geographic, social and management scales, sometimes referred to as the One-Plan approach (Conde et al., 2013), is increasingly being seen as an important framework for effective species conservation (Redford et al., 2012). It is underpinned by a need to understand, utilize and maintain the total genetic diversity available within all living members of a species, along with the possibility of contributions from biobanked material. Conservation genetic data should be available to inform both policy makers and wildlife managers in relation to a range of questions arising within an integrated approach, from extensive to intensive management. The list of applications for genetic data and management advice to species conservation is long and well established (Frankham et al. 2009), and a number of studies have started to

bridge traditional conservation domains through measurement of captive and wild population genetic diversity (Stanton et al., 2015; Milián-García et al., 2015; Hvilsom et al., 2013). However, comparative wild and captive molecular genetic data is still not generally applied across the spectrum of issues faced in conservation planning for a single species. In this paper we report on a study that integrates global evolutionary species history and subspecies diversity in the wild, with measures of founder diversity and forecasts of genetic variation within a captive conservation breeding programme.

1.1 The evolutionary and conservation status of the golden eagle in Japan

The golden eagle (*Aquila chrysaetos*) has six sub-species found across a circumpolar distribution in the temperate northern hemisphere. Due to its total population size (estimated at 300,000 individuals), the IUCN has classified this species as Least Concern (IUCN Red List 2013), however at the subspecies level and below, some populations of golden eagle are in severe decline.

The Japanese golden eagle (*A. c. japonica*) is one such sub-species, distributed only in Japan and possibly a part of the Korean Peninsula (Masuda et al., 1998). Unlike other sub-species, the Japanese golden eagle has adapted to heavily-forested mountainous areas, where its breeding success is considered to be related to the availability of older deciduous broad-leaved forests where large gaps in tree cover allow the eagles to hunt ground prey (Yui et al., 2005). Since 1981 the breeding success rate has decreased from 55.3 % to 24.6 % in Japan (The Society for Research of Golden Eagle, 2014) (Figure 1) and in 1995 the Japanese Ministry of the Environment classified this species as nationally endangered. The population size has been steadily decreasing since records began in 1981 (Figure A1) and there are now estimated to be only around 500 individuals in Japan (The Society for Research of Golden Eagle, 2014). Yui et al. (2005) suggested that reduced breeding may be associated with decreases of patchy forest cover and increases in overcrowded forest plantations. Inbreeding depression as a result of small population size has been also considered as a part of the reason for low breeding success, however there is no empirical evidence for this assertion and no studies to examine individual relatedness have been conducted. In an attempt to conserve the species, the Japanese Ministry of the Environment conducts ecological surveys, works to improve the environment around nest sites, and has commissioned conservation genetic analysis to inform management strategy. Despite

these efforts there is increasing concern over the long-term future of the species in Japan.

A global evolutionary genetic study of golden eagles by Nebel et al. (2015) using mtDNA control region sequencing revealed thirty haplotypes (maternal lineages) divided into two groups: Holarctic (21 types) and Mediterranean (9 types). The Japanese golden eagle belongs to the Holarctic group and, based on a small sample number, was found to be relatively diverse, displaying five haplotypes. Although three of these were found only in Japanese samples, the other two were shared across Eurasia and there was no clear phylogeographic relationship among them. The only previous study to focus on the Japanese golden eagle (Masuda et al. 1998) targeted the mtDNA pseudo-control region. This work also described five haplotypes however only two of these were confirmed to originate from known Japanese locations (the study included Korean, Chinese and unknown origin samples) and the two mtDNA data sets are not directly comparable, limiting their interpretation in relation to Japanese population diversity. However Masuda et al. also undertook a karyotypic study that suggests Japanese golden eagles display a different microchromosomal pattern to those previously observed at either the eastern or western ends of the Eurasian continent. This potentially supports a level of population distinctiveness for the Japanese golden eagle that would place constraints on future population recovery options.

1.2 Captive golden eagles in Japan

Given the known status of wild Japanese golden eagles, the Japanese Association of Zoos and Aquaria (JAZA) has acted to establish and manage a captive breeding programme from 1997. From a total of 16 founder birds, forty Japanese golden eagle individuals are alive in eight zoos. Seven founders are still living, with all live offspring derived from five of these seven (2014 studbook data) (Figure 2). To confound matters further, one breeding founder male that has given rise to ten living O1 offspring and two living O2 offspring is considered by zoos to potentially be of a different subspecies based on its unconfirmed origin and observed body size, and this entire family of thirteen birds has therefore been excluded from the breeding programme. This leaves a total of 27 live birds from which to breed. Within this group zoo-keepers are trying to minimize inbreeding by strategic mating based on

studbook information, but no genetic data is available to support current management and not all birds have successfully bred. In attempting to develop the captive population into a sustainable, representative insurance population for the Japanese golden eagle, research is required to understand their relationships and comparative diversity to the wild population, and to forecast their demographic and genetic trajectories.

1.3 Research objectives

The aim of this study was to conduct an integrated genetic analysis of wild and captive Japanese golden eagles to inform our understanding of sub-species population biology and support the management of the captive population down to the level of the individual. To achieve this we attempted to answer a number of questions relevant to applied conservation genetic management in Japan: 1. What levels of genetic diversity and population structure exist in wild Japanese golden eagles?; 2. To what extent has founder effect impacted the conservation value of the captive population?; 3. Are we able to inform individual management of wild and captive birds using molecular estimates of relatedness?; and 4. How can we sustain long-term genetic diversity and demographic viability of the captive population? These questions were addressed through undertaking phylogenetic, population genetic and individual level analyses, in combination with simulations of future population genetic change over time.

2. Materials and Methods:

2.1 Sampling

Fifty-one samples were obtained from the wild population (Iwate prefecture $n = 46$, Tochigi prefecture $n = 3$, Aomori prefecture $n = 2$) (Figure 3). Feather ($n = 41$), crop pellet ($n = 2$), egg membrane ($n = 2$), and faecal ($n = 1$) samples were collected under 18 nest sites between 1999 and 2014, with no repeated sampling of single nests across years. Muscle samples ($n = 4$) were collected from incidental carcasses, and a talon sample ($n = 1$) was collected from a private specimen. Twenty samples were obtained from the captive population including nine wild origin founder birds and eleven captive bred birds. Sixteen of these birds remain alive in captivity. Zoo keepers contributed to collect these samples (feather $n = 15$, blood $n = 5$). All samples were preserved at -20 °C until DNA extraction.

186

187 *2.2 DNA analysis*

188 Moulted feathers were processed to target the blood spot and basal tip for DNA
189 extraction, as the described by Horvath et al. (2005). All DNA extractions were
190 conducted using the Qiagen DNeasy Blood & Tissue Kit, or QIAamp DNA Stool
191 Mini Kit (Qiagen, GmbH, Hilden, Germany) following the manufacturers' protocols.

192

193 To investigate the evolutionary history and diversity of the *A. c. japonica* sub-species,
194 the Control Region (CR, D-loop) and pseudo Control Region (ψCR) of mtDNA were
195 sequenced. The CR primer pair amplify a 402 bp fragment, and primer sequences are
196 as follows: modGOEA_CR1L 5'-CCCCCGTATGTATTATTGTA-3' and
197 GOEA_CR595H 5'-GCAAGGTCGTAGGACTAACC-3' (Nebel et al., 2015). The
198 mtDNA ψCR, located in *A. c. japonica* between $tRNA^{Glu}$ and $tRNA^{Phe}$, was isolated
199 by Masuda et al. (1998), as the CR (Haring et al., 1999). The ψCR primer pair
200 amplify 444 bp fragment, and primer sequences are as follows: E-ACH 5'-
201 CTCTCCAAAATCTACGACCTGAA-3' and IE-ACH
202 5'CGTTGTAAACTTCAACTACAGAA-3' (Masuda et al., 1998). All PCR reactions
203 were conducted under the same conditions: a final volume of 10 µl, containing 1 µl
204 DNA, 5 µl Multiplex PCR Master Mix (Qiagen), 0.2 µM of forward and reverse
205 primer, 0.1 µg of T4 gene 32 Protein (Nippon Gene, Tokyo, Japan). The
206 thermocycling conditions were as follows: initial step of 94°C for 15 min; 45 cycles
207 of 94°C for 30s, 55°C for 45s, 72°C for 45s; and a final extension of 72°C for 30 min.
208 The High Pure PCR Product Purification Kit (Roche, Basel, Switzerland) was used to
209 purify PCR products. The Big Dye Terminator v3. 1 Cycle Sequencing Kit (Roche)
210 and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) were used to
211 determine nucleotide sequences.

212 Microsatellite genotyping was conducted to investigate genetic diversity using 16
213 loci derived from Japanese golden eagle (Sato et al., 2015). These microsatellite
214 markers were synthesized with fluorescent labels and amplified in three separate PCR
215 reactions. Multiplex 1 included AQJ03 (FAM), AQJ10 (FAM), AQJ30 (HEX),
216 AQJ36 (HEX), AQJ59 (NED). Multiplex 2 included AQJ08 (NED), AQJ27 (FAM),
217 AQJ49 (FAM), AQJ56 (HEX), AQJ66 (FAM). Multiplex 3 included AQJ19 (FAM),
218 AQJ28 (HEX), AQJ34 (HEX), AQJ40 (FAM), AQJ52 (NED), AQJ53 (FAM). PCR
219 reactions and conditions were the same as mtDNA analysis. The annealing

220 temperature of every multiplex set and cycle number was 55°C and 45 cycles.
221 Amplicon size was measured using an ABI PRISM 3130xl Genetic Analyzer (Applied
222 Biosystems), and genotypes were scored by eye with Peak Scanner Software 1.0
223 (Applied Biosystems). These PCR to genotyping steps were repeated three times for
224 each sample to control for allelic drop-out.
225
226 *2.3 Mitochondrial DNA analysis*
227 Mitochondrial CR, ψCR, and concatonated CR + ψCR sequences were determined
228 and aligned using MEGA 6.0 software (Tamura et al., 2013). Median-joining
229 networks for separate CR, ψCR, and CR + ψCR haplotype data were generated and
230 visualised using PopArt (http://popart.otago.ac.nz). Haplotype sequence diversities (*h*)
231 and haplotype richness (*hr*) were calculated using Contrib 1.4 (Petit et al., 1998) to
232 assess mtDNA genetic diversity within and among the sample localities. Where
233 informative, result data were combined with those of previous studies to create larger
234 datasets for re-analysis.
235
236 *2.4 Nuclear DNA analysis*
237 For the microsatellite data, the number of alleles (*Na*), observed (*Ho*) and expected
238 heterozygosities (*He*), and inbreeding coefficients (*F*) were calculated in GenAlEx
239 6.501 (Peakall and Smouse, 2006). In addition, deviation from Hardy-Weinberg
240 equilibrium (HWE) of wild samples was tested using GENEPOP on the web
241 (Raymond & Rousset, 1995). Allelic richness was calculated in HP-Rare (Kalinowski,
242 2005). Population genetic structure was investigated within the wild population and
243 between the wild and captive birds using Principle Coordinates Analysis (PCoA,
244 GenAlEx 6.501).. Evidence for a demographic bottleneck in the Iwate (wild)
245 population was examined using the programme BOTTLENECK (Cornuet and Luikart
246 1997) run under default parameters.
247
248 *2.5 Evaluation of molecular tools for kinship inference*
249 Given the completeness of the golden eagle captive pedigree, there was an
250 opportunity to examine the power of the DNA profiling system to validate familial
251 relationships and potentially infer pairwise kinship in captive or wild populations
252 where breeding data is not available. Probabilities of parental exclusion were
253 calculated to estimate the power to validate familial trios or parent-offspring pairwise

relationships (Jamieson & Taylor 1997, implemented in GenAlEx 6.501). Determining pairwise relatedness in the absence of pedigree data has been the subject of some debate in the *ex situ* conservation community, as traditional molecular relatedness coefficients that rely on accurate estimates of population allele frequencies are often confounded by non-random mating within captive populations (Ivy et al. 2016; Cabellero & Toro, 2002; Montgomery et al. 1997). The use of molecular co-ancestry to infer kinship, based on allele sharing among individuals, has been proposed as a more robust alternative and was implemented here using the program MolKin v.2 (Gutiérrez et al 2005). This approach was used to compare molecular estimates of co-ancestry against studbook records for twenty-two parent-offspring pairs, nineteen sibling pairs, twelve grandparent-grandchild, six avuncular pairs and assumed unrelated individuals, in order to evaluate the power of the DNA profiling system for assisting in pedigree reconstruction of both captive and wild birds.

2.6 Population Viability Analysis

Simulations were conducted to forecast the change in captive population size and genetic diversity under different scenarios over 200 years using Vortex 10 (Lacy and Pollak, 2015). First, biological and technical parameters were estimated from studbook data or information collected directly from zoo keepers (Table A1). Based on these fixed parameters, a series of preliminary simulations were run to determine the management conditions required to maintain population numbers and diversity from a founding size of 27 birds (the current breeding population) with known molecular genetic diversity data from twenty individuals (nuclear and mitochondrial DNA markers included). Three key breeding parameters that could be potentially subject to management intervention/control were identified and varied to explore a range of conditions under which the starting population could be sustained demographically and genetically: i) 'Maximum kinship within mate pair' enables management control of inbreeding but indirectly limits population growth where sufficiently unrelated birds are not available to breed. Varying this parameter ($x<k<0.25$) revealed that a conservatively low relatedness threshold, while desirable, led to rapid population extinction, resulting in a kinship maximum value of 0.125 being selected for future simulations. ii) 'Mate monopolization' refers to the proportion of adult males and females available to form breeding pairs which, in captivity, is largely under management control. The existing mate monopolization

rate was therefore adjusted based on an assumption that intensive management could increase the proportion of breeding adults and thereby enhance genetic diversity and reduce relatedness in subsequent generations. iii) The ability to supplement the captive population with wild sourced birds, or captive birds outside of the current breeding programme was also explored in order to maintain diversity and periodically add to the population size. For details of these simulated parameters see Table A1. The results of this preliminary simulation phase formed a range of management conditions under which a sustainable population was theoretically possible. Next, the actual management conditions and breeding restrictions currently in effect were used to forecast the future of the captive population and identify any issues relating to demographic or genetic sustainability (Table A1). Lastly, realistic modifications to management solutions were simulated in an attempt to mitigate declines in population size and diversity and develop a viable management strategy for the captive population of golden eagles in Japan. Supplementation from the wild of one male and one female at ten and two year intervals was simulated, reflecting a typical rate of wild bird rescue and a theoretical option for deliberate capture, respectively. Adjustments to mate monopolization were made to double the number of male and female birds breeding each year, from three pairs to six. While the focus of the PVA was on the sustainability of the captive breeding programme, simulated management solutions also included 'harvest' from the captive population to allow reinforcement of the wild population, thus establishing a preliminary model for reciprocal exchange of birds between wild and captive environments under a future one-plan approach.

3. Results:

3.1 Mitochondrial DNA analysis

From fifty-one wild samples, twenty-seven samples returned both reliable CR and ψCR sequences, and four samples had reliable ψCR sequences only. Wild samples displayed six CR haplotypes (H1, H2, H3, H4, H7, and H8) (Figure A2.1), with haplotype H1 observed in the Japanese subspecies for the first time, reinforcing the lack of Holarctic phylogeographic structure (Figure 4). At the pseudo-control region (ψCR), wild Japanese samples displayed three haplotypes (a, b, and d) (Figure A2.2), generating total of seven combined (CR + ψCR) mitochondrial sequence haplotypes (Figure A2.3). The distribution of these haplotypes in Japanese wild birds shows no

geographic pattern, with the most densely sampled region, Iwate, displaying nearly all observed mitochondrial lineages (Figure 3).

In the twenty captive samples, four samples failed to amplify both regions, but sixteen samples returned reliable sequences of both CR and ψCR. Captive samples displayed five CR haplotypes (H2, H3, H4, H7, and H8) (Figure A2.1) and the same three ψCR haplotypes as the wild birds (a, b, and d) (Figure A2.2), generating a total of six different combined (CR + ψCR) mitochondrial sequence haplotypes (Figure A2.3). Overall haplotype diversity (*h*) was marginally higher in the wild, which displayed three unique haplotypes compared to two in captivity, however this pattern reversed when rarefaction was applied to estimate haplotype richness (*hr*), accounting for variation in sample size (Table 1).

3.2 Nuclear DNA analysis

Thirty-nine wild samples and twenty captive samples were genotyped for the sixteen microsatellites. Individual DNA profiling revealed that all wild samples were collected from different individuals. In the wild population, an average of 75.6 % of loci were genotyped per individual, with 75.6 % of samples genotyped per locus. As expected, due to better sample quality, genotyping in the captive population was more successful with an average of 91.6 % of loci genotyped per individual and 91.6 % of samples genotyped per locus. Two of the sixteen loci (AQJ27 and AQJ30) showed significant heteroyzygote deficiency ($0.01<P< 0.05$). Across all loci, allelic richness and private allelic richness were approximately equal between wild and captive populations, while observed heterozygosity in the wild was slightly lower than in captivity (Table 2).

The results of PCoA analysis revealed no population genetic structuring of wild samples among or within the three different sampling areas (Iwate, Aomori and Tochigi) (Figure A3). Analysis of wild versus captive golden eagles showed no differentiation of the two groups (Figure A4), however, when the captive birds are divided into wild-rescued founders and birds bred in captivity, the captive bred group show a much more limited distribution of genetic variance.

Bottleneck analysis showed weak evidence of a recent loss in effective population size with significant heterozygote excess observed under the infinite allele model for all three tests ($0.01<P<0.05$), but not under the two-phase or stepwise mutation models of microsatellite mutation. There was no clear evidence of a mode-

355 shift from the expected L-shaped distribution of allele frequencies at mutation-drift
356 equilibrium (Figure A5).
357
358 *3.3 Evaluation of molecular tools for kinship inference*
359 Studbook verification via DNA parentage exclusion analysis within the Japanese
360 golden eagle population is achievable using the current 16-locus profiling system,
361 with exclusion probabilities ranging from 0.99310 to 0.99999, depending on the
362 particular scenario under consideration and the availability of parental samples. Co-
363 ancestry estimates within the captive population, including selfing relationships,
364 ranged from 0.18 to 0.91 (Table A3). While there is a clear trend of increasing
365 molecular kinship with known pedigree relationship (Figure 5), a large degree of
366 overlap was observed in molecular kinship among different levels of familial
367 relatedness that would severely limit the utility of this DNA profiling system for
368 pedigree reconstruction in captive or wild populations.
369
370 *3.4 Population Viability Analysis*
371 According to PVA results, the captive golden eagle population is unsustainable under
372 current management conditions, with simulations leading to population extinction
373 within 200 years (mean = 155 years) (Figure 6a). During this time continuous
374 decreases in population size were accompanied by significant losses in nuclear and
375 mitochondrial diversity.
376 Exploration of demographic and genetic forecasts with reduced mate-
377 monopolization (> proportion of adults breeding) leading to a marked increase in
378 population sustainability, with continuous population growth from n=27 to a mean of
379 over n=80 individuals in the first 100 years, as a greater proportion of birds
380 successfully bred (Figure 6b). However this was followed by a population collapse in
381 the second 100 years due to an increase in inbreeding reducing the number of
382 available mate pairs with mean kinship below the threshold of F=0.125, as indicated
383 by continual marked reductions in nuclear and mitochondrial genetic diversity in all
384 simulations (Figure 6b).
385 Individual supplementation from the wild every 10 years retarded loss of genetic
386 diversity but as a standalone intervention did not result in population growth (Figure
387 6c), as breeding rate could not achieve replacement levels. A combination of regular
388 supplementation and reduced mate-monopolization was successful over two hundred

years, with a stable mean population size of 95 birds (carrying capacity, n<100) retaining the majority of founder genetic diversity (Figure 6d).

Increasing individual supplementation from the wild to one pair every two years also resulted in a stable population, but at a slightly lower population number (n=80) (Figure 6e); however such supplementation is considered unrealistic without reciprocal wild releases ('harvest'). Simulated harvest at the same rate (one pair every two years) resulted in a sustainable genetic captive population but a demographic population that failed to increase in size (n=30) (Figure 6f).

4. Discussion:

As pressures on natural populations increase, so does the importance of conservation genetic management of both wild and captive populations. Addressing questions relating to diversity and inbreeding, founder effects and likely future retention of genetic variation is key to informing current and future best practice conservation management. To date, very few studies have been conducted that integrate empirical data on molecular genetic variation across *in situ* and *ex situ* populations, and that combine current genetic data with long-term demographic and genetic forecasts in zoo-based conservation breeding programmes. In this study we have demonstrated how these issues can be practically addressed through application of traditional molecular genetic tools to support an integrated approach to species conservation.

4.1. Genetic diversity and population structure in Japanese golden eagles

At a global level, as three of the six CR haplotypes observed in Japan have also been observed across Eurasia, there is no clear evidence that the Japanese golden eagle is evolutionarily distinct from other populations. This is perhaps not surprising given the possibility that golden eagles may be able to migrate from Japan to continental Asia (Masuda et al. 1998). However previous karyotype results indicating that the Japanese subspecies may display a different number of microchromosomes compared to its continental conspecifers (Masuda et al. 1998), does raise the possibility that nuclear differentiation has occurred. This should be further investigated before drawing any conclusions on the relative evolutionary and conservation significance of the Japanese golden eagle, or prior to considering reinforcement of the Japanese population from the continent.

Within Japan, the mtDNA haplotype results show that Japanese golden eagles are composed of multiple lineages and are diverse in the context of the global population, displaying 35% of all known holarctic CR haplotypes; more than any other geographic region described by Nebel et al. (2015). Taken alongside the observation of additional pseudo-control region haplotypes, these findings suggest that the Japanese subspecies maintains higher diversity than previously thought (Masuda et al. 1998), despite a declining population. Within Japan, the lack of phylogeographic structure, as evidenced by the same haplotypes being observed at either end of their distribution (Figure 3), is also likely to reflect the high dispersal ability of golden eagles and this has probably helped to retain species diversity and avoid lineage extirpation that affects less mobile species with more highly structured distributions. Based on the current data, there is no evidence of need for a regional management approach to golden eagle conservation within Japan. Further geographic sampling would help to validate this finding and enable the nuclear data from Iwate to be used within a Japan-wide evaluation of population genetic structure. A similar conclusion was drawn for golden eagles in mainland Scotland, UK (Ogden et al. 2015), where much larger sample sizes over a similar area revealed a lack of population structure using nuclear DNA microsatellites and a recommendation for management as a single population unit.

The pseudo control region has been previously detected only in Picidae, Cuculidae, Falconidae, Accipitridae, and the suboscines group of Passeriformes (Mindell et al., 1998; Haring et al., 1999). The use of both pseudo and true control regions provides additional resolution with which to assess lineage diversity of these species. Within our study, the combination of control region and pseudo control region sequence data also allowed the integration of different published datasets. In Iwate prefecture, nine mitochondrial (concatenated CR + ψCR) haplotypes were recorded from samples collected at only thirteen nest sites, suggesting that a relatively high number of female lineages persist in the region. Such results are consistent with low female dispersal and indicate that the population has not yet entered a genetic bottleneck during which haplotype diversity would be expected to drop and nuclear diversity would show an elevated level of heterozygosity relative to allelic diversity, which was not observed in our microsatellite dataset.

4.2. Assessing founder effect in captive Japanese golden eagles

It is relatively unusual to have the opportunity to directly compare genetic diversity in a declining natural population with that present in a recently established captive breeding programme. Comparative analysis of the wild and captive (zoo) populations indicated roughly equivalent levels of diversity and no differentiation between the two groups , suggesting that the current breeding programme encompasses most of the extant genetic variation in the Japanese golden eagle observed to date. This is a positive finding in terms of the establishment of an insurance population for the Japanese subspecies, and is likely due to the fact that founder individuals were captured from all over Honshu island (Akita prefecture, Miyagi prefecture, Niigata Prefecture, and Fukui prefecture (Figure 2). While it could be argued that these results also give rise to concern about the likely loss of diversity in the wild over the past 50 years, there was no evidence of a strong nuclear genetic bottleneck having occurred, at least in the Iwate region. Nevertheless, despite small samples being collected from a further seven localities from northern to southern Honshu island, our samples were mainly collected from Iwate prefecture and temporal samples are not available for comparison, therefore it is not possible to be certain from the current dataset that diversity is not being lost over time in other areas of Japan.

Importantly, our findings do suggest that founder effects, caused by creating a captive population using only a fraction of wild population diversity, will have been minimized. Founder effects are a key concern in *ex situ* conservation management but have until now been treated as an 'elephant in the room', with studbook breeding programmes defining 100% gene diversity as being the diversity contained within the founders, irrespective of whether those founders represent genetic diversity in the species as a whole. This can lead to somewhat spurious studbook measures of (theoretical) genetic diversity, where for example in zebras, captive populations with high studbook gene diversity have significantly lower evolutionary molecular genetic diversity than populations with much lower studbook diversity (Ito et al. 2017). The ability to quantitatively compare molecular genetic diversity in wild source and zoo founder populations provides conservation managers with much more detailed and accurate information with which to plan long-term species conservation measures. Previous studies of this nature have typically recorded reduced mitochondrial DNA diversity but similar levels of nuclear microsatellite DNA diversity (Stanton et al, 2015; Muñoz-fuentes et al, 2008, Shen et al.2009; McGreevy et al. 2011) in captivity,

suggesting that while captive populations may largely be avoiding significant genetic loss in captivity, their founders do not represent wild lineage diversity. Our findings indicate that from a Japanese perspective, the founders are more representative of the extant wild population, although more comprehensive geographic sampling is required to confirm this.

4.3. Individual level molecular genetic management through pedigree reconstruction

Verification of familial relatedness is a well-known issue in studbook-based breeding programmes and the application of molecular data to resolve pedigree gaps or reconstruct entire pedigrees has been employed to address this problem (Fienieg and Galbusera 2013). The approach used here demonstrates the power of DNA parentage approaches to verify studbook pedigree relationships in the Japanese golden eagle, however it has limited power to reconstruct multi-generational pedigrees *de novo*. In wild populations, use of pedigrees to investigate population genetic processes relevant to conservation, such as inbreeding depression, is considered superior to using indirect estimators of pairwise relatedness or co-ancestry based on molecular markers (Pemberton 2008). In the absence of a known wild pedigree one solution would be to peform a two-step process whereby molecular co-ancestry estimates are used alongside DNA parentage/sibship analysis to reconstruct a pedigree, which is subsequently used to directly calculate pairwise relatedness within the population. Achieving molecular pedigree reconstruction is likely to require significantly larger numbers of molecular markers (Ivy et al. 2016), but if achieved for the wild Japanese golden eagles, would offer significant insights into conservation relevant demographic and genetic processes in this declining population. To this end a study to generate thousands of genome-wide SNP markers in the Japanese golden eagle is now underway.

4.4. Sustaining captive genetic diversity and demographic viability

Our results suggest that genetic diversity within the captive population represents a high proportion of extant wild genetic diversity. However, it has not been possible within this study to evaluate historic Japanese diversity and, while it may appear that the captive breeding programme has avoided a severe founder effect, it is clear from the pedigree data that the early generations of breeding have led to an extreme skew in founder representation that threatens to decimate the genetic diversity of the captive

population over the next few decades. This is further evidenced by the distribution of genetic variance in the captive population under PCoA, with the distribution of birds actually bred in captivity, as opposed to founder individuals, displaying a much-reduced proportion of total observed genetic variation. The current captive population is essentially comprised of one big family (including 1 founder bird) and 5 non-paired founders. The current exclusion from the breeding programme of descendants from a questioned male (Figure 2) is not supported by its mitochondrial or nuclear DNA data, nor are there any signs of outbreeding depression in its offspring, and a karyotypic analysis of this individual bird is urgently recommended to inform a decision regarding the inclusion of this potentially genetically valuable family.

Successive generations will inevitably see further rapid loss of genetic diversity and increases in inbreeding, with PVA indicating terminal decline under current management conditions. The key issue in our study was the imposition on the PVA of a maximum pairwise kinship value to form a breeding pair (F=0.125); once this threshold was reached, breeding ceased. This may be considered an artificial restriction to population survival, however the alternative is to continue breeding increasingly related birds, a process that rapidly leads to loss of diversity and increased risks of inbreeding, significantly reducing the conservation value of the captive population. Indeed, the constraint on relatedness of breeding pairs did appear to successfully prevent an increase of lethal alleles in all VORTEX simulations, which would have been indicative of inbreeding depression, although such a strategy creates a trade-off between inbreeding and population growth.

Our simulations yielded a solution that would require increasing the proportion of individual birds breeding, as well as supplementation from outside the programme. At present only three males and three females are selected to breed annually and breeding success for many pairs is relatively low. Increasing the number of pairings would involve greater institutional exchange of birds (or gametes for AI); increasing breeding success might be achieved through modifications to husbandry practices. Both routes to increased reproduction could be attempted but would need concerted management effort. In terms of supplementation, from 1970 to 2014 fifteen wild rescue birds were included into the captive population suggesting that supplementation with a pair of birds every ten years may be achievable through an

559 opportunistic approach. Increasing the frequency of supplementation to the levels
560 required for a sustainable captive population (one pair every two years), without
561 manipulating mate monopolization, was shown to be possible, however this could
562 only be achieved through the deliberate removal of Japanese birds from the wild, or
563 importation of birds from outside Japan; both options would require a number of
564 biological and legal challenges to be overcome.
565
566 In the context of managing genetic diversity, supplementation of the captive
567 population represents a form of genetic rescue (short term) or genetic restoration (long
568 term) (Weeks *et al*. 2011). Although such terms were devised to describe
569 conservation interventions in natural populations, a vision of integrated *in situ* / *ex situ*
570 conservation management should allow for either type of population to benefit from
571 augmentation of genetic diversity. Genetic rescue has been previously demonstrated
572 to be effective in small population conservation (Frankham 2015) and its inclusion in
573 PVA simulations has previously been applied to other species (Harrison *et al.* 2016),
574 suggesting there is certainly scope to explore these ideas further in the Japanese
575 golden eagle. Wild capture, combined with wild release, as a form of ongoing genetic
576 exchange under full implementation of the one-plan approach, perhaps offers the best
577 solution for maintaining an integrated *in-situ / ex-situ* conservation population of
578 golden eagles in Japan. However, the initial models examined here suggested that the
579 captive population is very sensitive to harvest suggesting that management planning
580 would benefit from additional comprehensive simulation work to deliver
581 recommendations for sustainable rates of reciprocal harvest / supplementation. While
582 the timescale of simulated declines is relatively long (>100 years), due principally to
583 the life history of the species, it is clear that intervention should be considered and this
584 will be most effective if implemented immediately.
585
586 The application of PVA approaches such as Vortex have typically been to wild
587 population demographic simulation, with consideration given to supplementation
588 from captivity; here we are effectively reversing this application. Few other examples
589 exist, although a similar approach taken toward the European captive eastern black
590 rhino also highlighted the effects of strong reproductive skews on the long-term
591 demographic and genetic sustainability of small closed populations (Edwards et al.
592 2015), suggesting that population viability analysis of captive breeding programmes

should be considered more widely. The suggestion that non-intervention may lead to population extinction in Japanese golden eagles is clearly important and may have broad implications for other captive breeding programmes. Similar findings were made by Suter et al. (2014) who examined the captive Asian elephant population in Laos and concluded that its long-term viability is compromised under current management conditions. The removal of animals from the wild to supplement captive breeding programmes is often contentious and requires careful justification. However if captive populations are really to form reservoirs or insurance populations to support the survival of species, then it is important that their genetic diversity is representative of extant variation and that this diversity is actively managed over the long term.

4.5 Management Recommendations

Based on the combined findings of this study, a number of management recommendations for the Japanese golden eagle can be made:

- The gradual but continuous decline in wild Japanese golden eagle numbers gives importance to the captive population as a conservation resource, justifying ongoing intensive management.
- Despite a genetically diverse founder base, under current projections a large proportion of diversity will be lost in the near future threatening the sustainability of the captive population and necessitating changes to management practice.
- To maintain the diversity of the captive population it should be supplemented with additional, unrelated individuals. Options for supplementary birds may include captive birds currently excluded from the breeding programme, wild Japanese birds, or birds from outside Japan.
- Genetic data suggest that all three options would be compatible with the evolutionary history of the species in Japan, however further investigations of karyotype (chromosomal make-up) and morphological differentiation should be performed to increase confidence in these findings.
- To create an integrated population management solution for the Japanese golden eagle, a model of reciprocal exchange between the wild and captive populations should be considered, with the aim of managing the number and genetic diversity of birds in both groups.

- Initial simulations indicate that supplementation of the captive population with birds every ten years combined with improved reproductive success would achieve sustainability; however, further Population Viability Analysis for wild and captive birds is recommended to test and develop alternative practical solutions.

4.6 Conclusions

Future approaches to biological conservation will need to maximise the use of all available sources of biological diversity, from pristine wilderness to cryo-preserved biobanks. Understanding how these natural resources relate to one another and integrating them within conservation programmes will require the development of continuous population management systems, for which genetic data will likely act as a common currency. For the Japanese golden eagle, such an approach has demonstrated how its conservation can be informed by simultaneous assessments of wild and captive genetic diversity.

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788 ## 7. Appendix

789 An appendix containing supplementary tables (Tables A1-A3) and figures
790 (Figures A1-A5) is available online.

8. Tables:

Table 1: Haplotype diversity (*h*) and haplotype richness (*Hr*) of CR, ψCR, and concatenated CR + ψCR of mtDNA, showing broadly similar levels of genetic variation in captive and wild golden eagles.

		N	*h*	*unique*	*se*	*hr*
CR	wild	27	0.746	1	0.062	3.36
	captive	16	0.667	0	0.113	3.09
ψCR	wild	31	0.239	0	0.096	1.42
	captive	16	0.342	0	0.140	2.00
CR + ψCR	wild	27	0.764	3	0.067	4.91
	captive	16	0.733	2	0.102	5.00

N, Number of samples; *h*, Haplotype diversity; *unique*, unique haplotypes; *se*, Standard error of *h*; *hr*, haplotype richness (rarefied)

Table 2: Genetic diversity indices for sixteen microsatellite markers showing similarity between levels of molecular diversity between current wild (Iwate) and captive populations.

	N	*Na*	*Np*	*Ar*	*PAr*	*Ho*	*He*	*F*
wild	39	4.4	16	3.37	0.65	0.519	0.560	0.08
captive	20	4.1	10	3.40	0.68	0.590	0.550	-0.07

N, nmber of samples; *Na,* number of Different Alleles; *Np*, private alleles; *Ar*, allelic richness; *PAr*, private allelic richness; *Ho*, observed heterozygosity; *He*, expected heterozygosity; *F*, inbreeding coefficient

9. Figures:

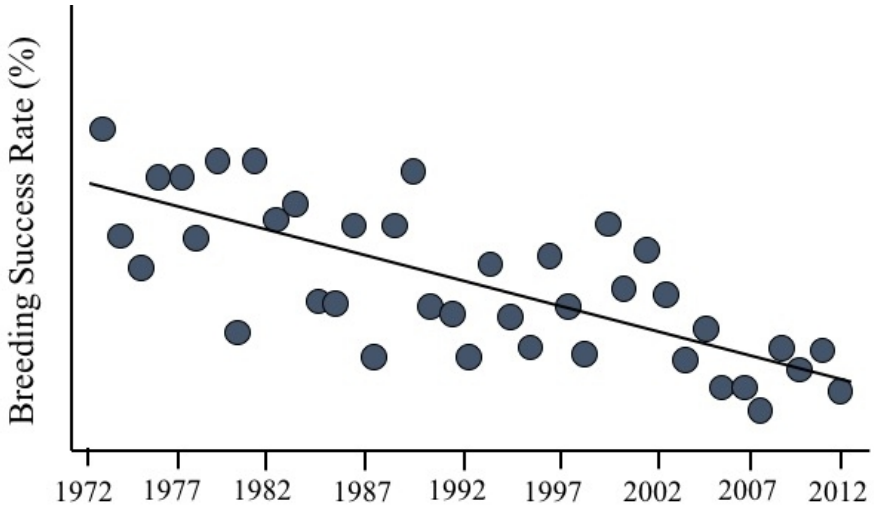

Figure 1 The breeding success rate of Japanese golden eagle in Iwate-prefecture from 1972 to 2012. The success rate has gradually decreased from around 40% in the 1970's to around 10% in the 2000's.

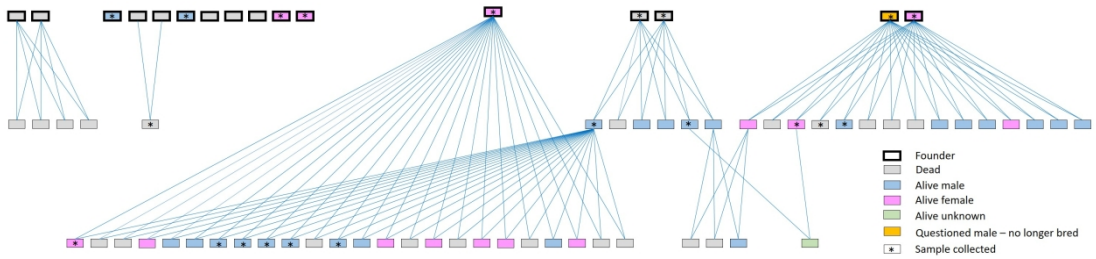

Figure 2 The kinship tree of captive golden eagles in Japan, created from 2014 studbook data for a total of 40 living individuals, showing the current severe skew in breeding contribution. There were 16 founder birds, seven of which are still living. Grey = dead, blue = living males, pink = living females. One breeding founder male (orange) is considered as potentially a different subspecies (morphological variation), and all his descendants (12 offspring) are currently excluded from the breeding program.

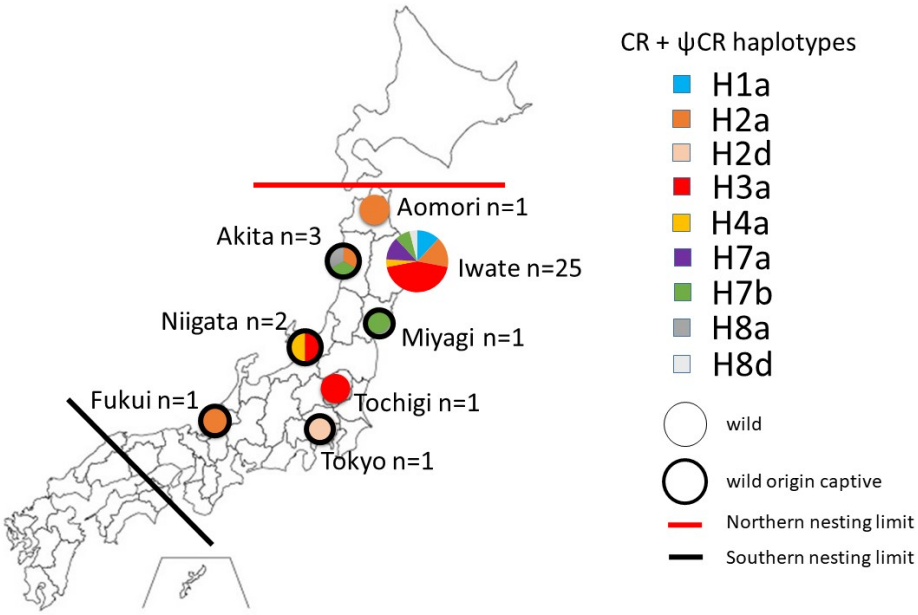

Figure 3 The map of Japan with all mtDNA haplotypes of concatenated CR + ψCR observed in wild origin individuals. There are nine haplotypes. Haplotypes H2d and H8a were only found only in wild-origin captive birds. Individuals from Iwate-prefecture (n = 25) display seven haplotypes.

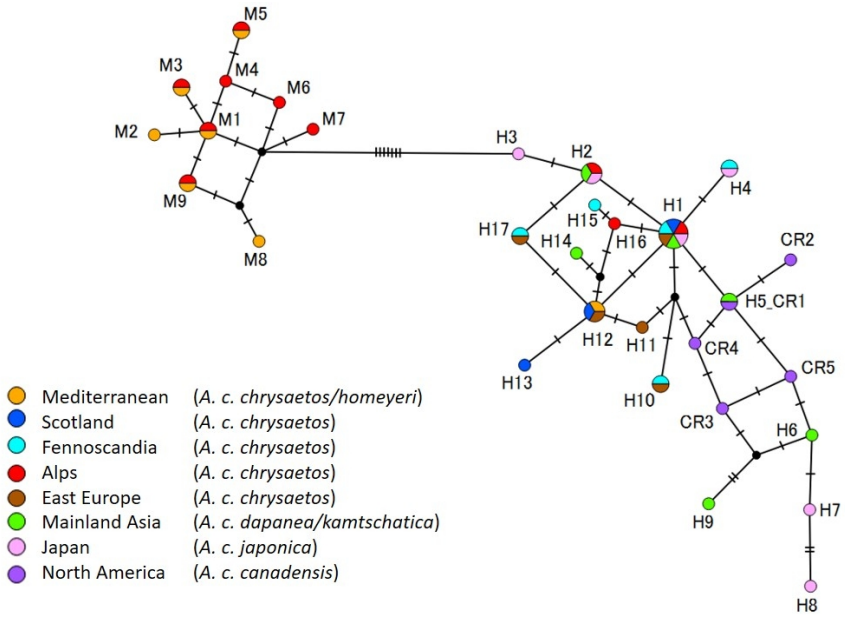

Figure 4 Median-joining network of the mtDNA control region haplotypes. 30 haplotypes are distributed globally in two lineages (haplotypes M, and haplotypes H/CR) (after Nebel et al., 2015). H1 is found in six areas including Japan. Six haplotypes (H1, H2, H3, H4, H7, and H8) are observed in Japan, three of which are currently unique to the country.

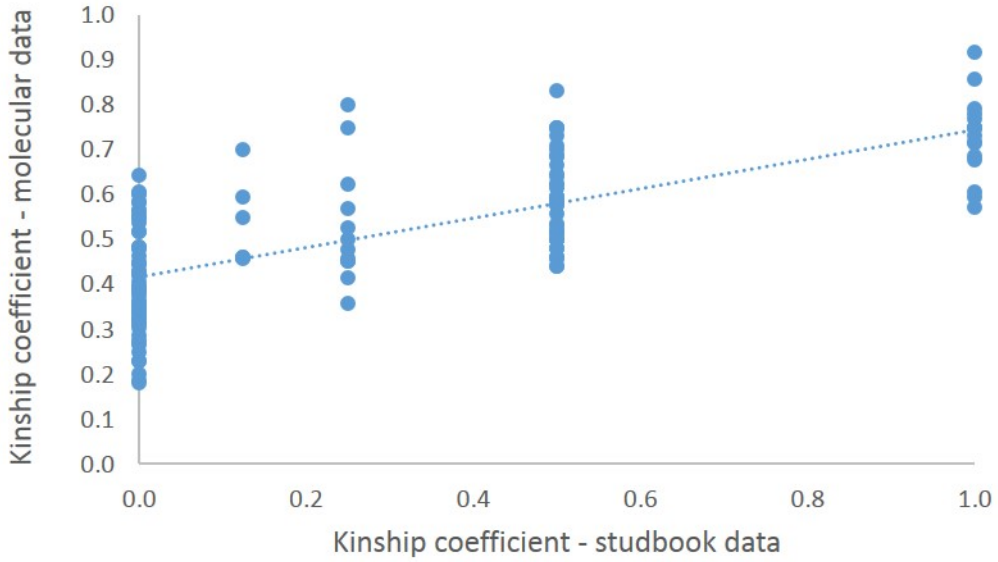

Figure 5 Distribution of pairwise kinship coefficients in captive related individuals extracted from studbook (horizontal axis), and calculated from molecular profiling (vertical axis) by MolKin v.2. Coefficients of between self, parent-offspring or siblings, grandparent-grandchild, avuncular, and non-related pairs were defined as 1.0, 0.5, 0.25, 0.125, and 0 on the horizontal axis. The spread of molecular kinksip coefficients for pairs within each relationship category resulted in only a weak correlation between the datasets, limiting the utility of this DNA marker system for predicting pedigree relationships.

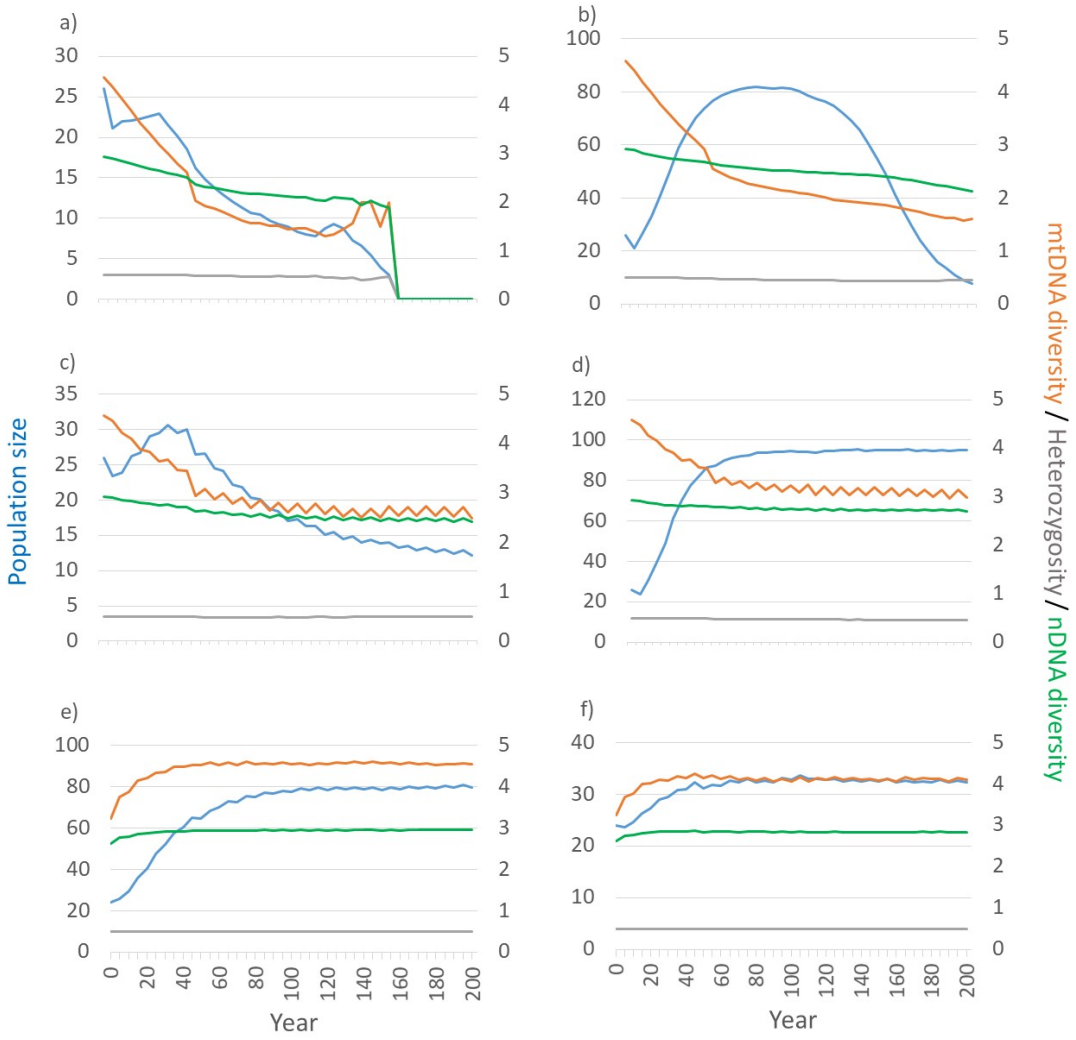

Figure 6 Vortex simulation results under six different scenarios: a) actual population parameters based on current management; b) increase in the proportion of breeders (18% to 36% in males, and 25% to 50% in females); c) supplementation with two unrelated individuals (1 male: 1 female) every 10 years; d) a combination of 'b' and 'c'; e) supplementation with two unrelated individuals (1 male: 1 female) every 2 years; and f) supplementation (1 male: 1 female) and removal (to wild) of two individuals (1 male: 1 female) every 2 years. Population size (the blue line) is shown on the left hand axes; number of mtDNA haplotypes (orange), nuclear heterozygosity (grey), and number of nuclear DNA alleles (green) are on the right hand axes. Scenarios a) to c) show population collapse; scenarios d) to f) suggest sustainable populations over 200 years.

Appendix

Supplemental Tables

Table A1 Description of PVA parameters used in the different simulations

1. Simulations were run with a common set of Base Settings for the following parameters: No. of iterations, Extinction, Inbreeding depression, EV correlation, Catastrophe events and Carrying capacity (estimated as current capacity in Japanese zoos).
2. Biological parameters were estimated based on known species biology with modifications for the captive population based on consultation with zoo-keepers. The reproductive system was 'Polygynous' (unlike the natural system) because studbook managers can adjust breeding pairs annually. The reproductive period of both male and female is 5 years old to 30 years old (estimated from studbook), and maximum life span was forty-five years old (as recorded in studbook). Maximum number of broods per year and progeny per year are two, and the sex ratio at birth is 50%. The mortality rate from age 0 to 1 is 20%, 1 to 2, 2 to 3, 3 to 4, 4 to 5, annual mortality after age 5 are 5% (estimated from studbook). There are no catastrophes and no harvests. In order to prevent serious inbreeding, $F < 0.125$ was selected based on a series of trial simulations from F=0.03 to F=0.25 (not shown). The frequencies of 16 microsatellite loci and CR haplotypes were used to estimate heterozygosity, the number of alleles per microsatellite and the number of mtDNA CR haplotypes. The mean age in the initial population, taken directly from studbook records, was 11.4 years old in males (17 individuals: one age1, one age 4, two age 5, two age 6, two age 7, three age 8, one age 12, one age 14, one age 15, one age 16, one age 27, and one age 45) and 9.7 years old in female (10 individuals: one age 1, one age 2, two age 3, one age 4, one age 8, one age 11, one age 15, one age 20, and one age 30).
3. This set of fixed parameters (1. and 2. above) was then used as the basis for conducting simulations of the actual population under current management conditions with Actual Population Parameters (column 2) for: Maximum kinship within a mate, annual supplementation, male mate monopolization (%) and female mate monopolization (%). The results of these simulations are shown at the foot of column 2 (survival and loss of genetic diversity

measures). All population simulations went extinct within 200 years (Figure 6a).

4. Lastly these population parameters were adjusted within realistic limits in an attempt to identify more sustainable management strategies for maintaining genetic diversity and population numbers (column 3). Single parameter adjustments improved simulation outputs (Figures 6b and 6c), however a combination of parameter adjustments was required generate a long-term sustainable solution (Figure 6d). Further simulations to increase supplementation rate (2 birds every 2 years) and to assess the effects of harvesting from the captive population for wild release were also simulated (Figure 6e & 6f), but neither scenario is considered a current practical option.

Vortex Parameter (Inputs to or outputs from the simulation)			**Actual population parameters**	**Suggested management solution**
Base settings	Iterations / time steps		1,000/200 years	1,000/200 years
	Extinction		Only one sex remaining	Only one sex remaining
	Inbreeding depression		6.29 (default)	6.29 (default)
	EV correlation		0	0
	Catastrophe events		0	0
	Carrying capacity		100 individuals	100 individuals
Fixed (Biological or practical limitations)	Reproductive system		Polygynous	Polygynous
	Founder age / sex		Known-see table legend	Known-see table legend
	Max. lifespan		45 years	45 years
	Reproductive period		5 to 30 years old	5 to 30 years old
	Max. broods per year		2	2
	Max progeny per year		2	2
	Distribution of broods per year (%)	0	79	79
		1	12.5	12.5
		2	8.5	8.5
	Mortality rate (%)	Age 0 to 1	20	20
		1 to 2	5	5
		2 to 3	5	5
		3 to 4	5	5
		4 to 5	5	5
		After age 5	5	5
Controllable (potentially subject to management)	Maximum kinship within mate pair		0.125	0.125
	Annual suppl. from wild (10 years intervals)		0	1 m, 1 f
	Male mate monopolization (%)		18	36
	Female mate monopolization (%)		25	50
Output measures (Simulation results)	No. birds surviving at 100 years		8.94	94.03
	No. birds surviving at 200 years		0 (extinction)	94.95
	% loss nuclear DNA diversity at 100 years		10.6	7.8
	% loss nuclear DNA diversity at 200 years		100 (extinction)	9.2
	% loss mtDNA diversity at 100 years		68.5	32.8
	% loss mtDNA diversity at 200 years		100 (extinction)	34.9

Table A2 Pairwise kinship coefficients between captive individuals estimated from studbook (studbook kinship), and calculated from molecular profiling (Molecular kinship) by MolKin v.2.

Bird 1	Bird 2	Relationship	Studbook kinship	Molecular kinship
ZGE001	ZGE001	self	1.0	0.719
ZGE001	ZGE004	non-related	0.0	0.517
ZGE001	ZGE005	non-related	0.0	0.286
ZGE001	ZGE007	non-related	0.0	0.357
ZGE001	ZGE008	siblings	0.5	0.500
ZGE001	ZGE011	siblings	0.5	0.688
ZGE001	ZGE013	non-related	0.0	0.554
ZGE001	ZGE014	siblings	0.5	0.625
ZGE001	ZGE015	siblings	0.5	0.594
ZGE001	ZGE016	siblings	0.5	0.750
ZGE001	ZGE017	non-related	0.0	0.339
ZGE001	ZGE018	grandparent-grandchild	0.25	0.458
ZGE001	ZGE020	uncle-nephew	0.125	0.462
ZGE001	ZGE024	grandparent-grandchild	0.25	0.453
ZGE001	ZGE1921-f	parent-offspring	0.5	0.641
ZGE001	ZGE1921-m	parent-offspring	0.5	0.500
ZGE004	ZGE004	self	1.0	0.733
ZGE004	ZGE005	non-related	0.0	0.327
ZGE004	ZGE007	parent-offspring	0.5	0.558
ZGE004	ZGE008	non-related	0.0	0.483
ZGE004	ZGE011	non-related	0.0	0.545
ZGE004	ZGE013	parent-offspring	0.5	0.596
ZGE004	ZGE014	non-related	0.0	0.385
ZGE004	ZGE015	non-related	0.0	0.567
ZGE004	ZGE016	non-related	0.0	0.583
ZGE004	ZGE017	parent-offspring	0.5	0.462
ZGE004	ZGE018	non-related	0.0	0.313
ZGE004	ZGE020	non-related	0.0	0.538
ZGE004	ZGE024	non-related	0.0	0.600
ZGE004	ZGE1921-f	non-related	0.0	0.517
ZGE004	ZGE1921-m	non-related	0.0	0.483
ZGE005	ZGE005	self	1.0	0.679
ZGE005	ZGE007	parent-offspring	0.5	0.442
ZGE005	ZGE008	non-related	0.0	0.268
ZGE005	ZGE011	non-related	0.0	0.275
ZGE005	ZGE013	parent-offspring	0.5	0.458
ZGE005	ZGE014	non-related	0.0	0.229
ZGE005	ZGE015	non-related	0.0	0.357
ZGE005	ZGE016	non-related	0.0	0.313
ZGE005	ZGE017	parent-offspring	0.5	0.481
ZGE005	ZGE018	non-related	0.0	0.182
ZGE005	ZGE020	non-related	0.0	0.271
ZGE005	ZGE024	non-related	0.0	0.339
ZGE005	ZGE1921-f	non-related	0.0	0.321
ZGE005	ZGE1921-m	non-related	0.0	0.304
ZGE007	ZGE007	self	1.0	0.607
ZGE007	ZGE008	non-related	0.0	0.339

ZGE007	ZGE011	non-related	0.0	0.350
ZGE007	ZGE013	siblings	0.5	0.500
ZGE007	ZGE014	non-related	0.0	0.188
ZGE007	ZGE015	non-related	0.0	0.429
ZGE007	ZGE016	non-related	0.0	0.350
ZGE007	ZGE017	siblings	0.5	0.442
ZGE007	ZGE018	non-related	0.0	0.200
ZGE007	ZGE020	non-related	0.0	0.386
ZGE007	ZGE024	non-related	0.0	0.446
ZGE007	ZGE1921-f	non-related	0.0	0.321
ZGE007	ZGE1921-m	non-related	0.0	0.375
ZGE008	ZGE008	self	1.0	0.594
ZGE008	ZGE011	siblings	0.5	0.583
ZGE008	ZGE013	non-related	0.0	0.464
ZGE008	ZGE014	siblings	0.5	0.482
ZGE008	ZGE015	siblings	0.5	0.516
ZGE008	ZGE016	siblings	0.5	0.667
ZGE008	ZGE017	non-related	0.0	0.339
ZGE008	ZGE018	grandparent-grandchild	0.25	0.417
ZGE008	ZGE020	uncle-nephew	0.125	0.462
ZGE008	ZGE024	grandparent-grandchild	0.25	0.453
ZGE008	ZGE1921-f	parent-offspring	0.5	0.516
ZGE008	ZGE1921-m	parent-offspring	0.5	0.531
ZGE011	ZGE011	self	1.0	0.792
ZGE011	ZGE013	non-related	0.0	0.604
ZGE011	ZGE014	siblings	0.5	0.688
ZGE011	ZGE015	siblings	0.5	0.708
ZGE011	ZGE016	siblings	0.5	0.700
ZGE011	ZGE017	non-related	0.0	0.364
ZGE011	ZGE018	grandparent-grandchild	0.25	0.528
ZGE011	ZGE020	uncle-nephew	0.125	0.550
ZGE011	ZGE024	grandparent-grandchild	0.25	0.500
ZGE011	ZGE1921-f	parent-offspring	0.5	0.646
ZGE011	ZGE1921-m	parent-offspring	0.5	0.583
ZGE013	ZGE013	seif	1.0	0.714
ZGE013	ZGE014	non-related	0.0	0.481
ZGE013	ZGE015	non-related	0.0	0.643
ZGE013	ZGE016	non-related	0.0	0.583
ZGE013	ZGE017	siblings	0.5	0.462
ZGE013	ZGE018	non-related	0.0	0.386
ZGE013	ZGE020	non-related	0.0	0.563
ZGE013	ZGE024	non-related	0.0	0.607
ZGE013	ZGE1921-f	non-related	0.0	0.482
ZGE013	ZGE1921-m	non-related	0.0	0.554
ZGE014	ZGE014	self	1.0	0.857
ZGE014	ZGE015	siblings	0.5	0.536
ZGE014	ZGE016	siblings	0.5	0.583
ZGE014	ZGE017	non-related	0.0	0.231
ZGE014	ZGE018	grandparent-grandchild	0.25	0.568
ZGE014	ZGE020	uncle-nephew	0.125	0.458
ZGE014	ZGE024	grandparent-grandchild	0.25	0.357
ZGE014	ZGE1921-f	parent-offspring	0.5	0.625
ZGE014	ZGE1921-m	parent-offspring	0.5	0.518
ZGE015	ZGE015	self	1.0	0.750
ZGE015	ZGE016	siblings	0.5	0.833

ZGE015	ZGE017	non-related	0.0	0.446
ZGE015	ZGE018	grandparent-grandchild	0.25	0.479
ZGE015	ZGE020	uncle-nephew	0.125	0.596
ZGE015	ZGE024	grandparent-grandchild	0.25	0.625
ZGE015	ZGE1921-f	parent-offspring	0.5	0.578
ZGE015	ZGE1921-m	parent-offspring	0.5	0.578
ZGE016	ZGE016	self	1.0	0.917
ZGE016	ZGE017	non-related	0.0	0.450
ZGE016	ZGE018	grandparent-grandchild	0.25	0.800
ZGE016	ZGE020	uncle-nephew	0.125	0.700
ZGE016	ZGE024	grandparent-grandchild	0.25	0.750
ZGE016	ZGE1921-f	parent-offspring	0.5	0.750
ZGE016	ZGE1921-m	parent-offspring	0.5	0.750
ZGE017	ZGE017	self	1.0	0.571
ZGE017	ZGE018	non-related	0.0	0.250
ZGE017	ZGE020	non-related	0.0	0.396
ZGE017	ZGE024	non-related	0.0	0.429
ZGE017	ZGE1921-f	non-related	0.0	0.321
ZGE017	ZGE1921-m	non-related	0.0	0.393
ZGE018	ZGE018	self	1.0	0.750
ZGE018	ZGE020	parent-offspring	0.5	0.500
ZGE018	ZGE024	non-related	0.0	0.396
ZGE018	ZGE1921-f	non-related	0.0	0.396
ZGE018	ZGE1921-m	parent-offspring	0.5	0.521
ZGE020	ZGE020	self	1.0	0.769
ZGE020	ZGE024	parent-offspring	0.5	0.731
ZGE020	ZGE1921-f	non-related	0.0	0.423
ZGE020	ZGE1921-m	siblings	0.5	0.615
ZGE024	ZGE024	self	1.0	0.781
ZGE024	ZGE1921-f	non-related	0.0	0.422
ZGE024	ZGE1921-m	parent-offspring	0.5	0.594
ZGE1921-f	ZGE1921-f	self	1.0	0.750
ZGE1921-f	ZGE1921-m	non-related	0.0	0.406
ZGE1921-m	ZGE1921-m	self	1.0	0.688

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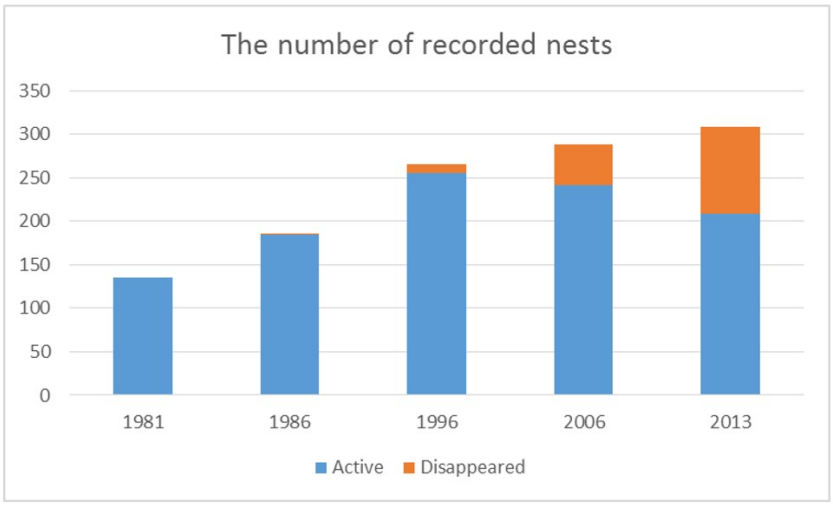
Supplemental Figures



The Society for Research of Golden Eagle (2015)

Figure A1 The number of recorded golden eagle pairs in Japan (modified from The Society for Research of Golden Eagle., 2015). The blue colour signifies active (observed) pairs, orange signifies pairs that have disappeared since the start of surveying. The total number of pairs increased from the 1970's as the survey expanded. An empty nest was founded in 1986 for the first time, and the number of missing pairs has steadily increased. In total, 99 pairs have disappeared from 1986 to 2013 and the total number of pairs is now dropping.

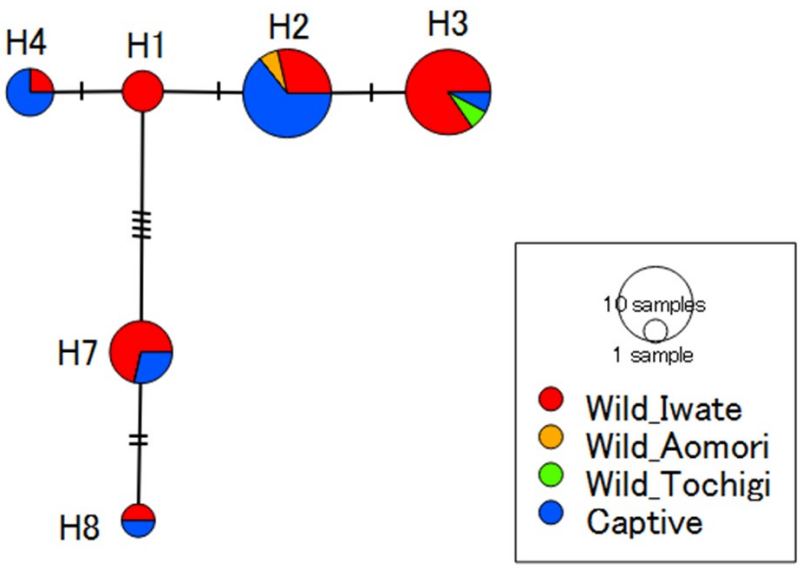


Figure A2.1 Median-joining network of CR haplotypes founded in Japan. It contains 6 haplotypes observed in 27 wild individuals and 16 captive individuals. Red colour indicates haplotypes observed in the wild Iwate-prefecture, orange in the wild Aomori-prefecture, green in the wild Tochigi-prefecture, and blue found in captive population. The circle size indicates the number of samples of each haplotype, and the number of dashes between each haplotypes means the number of nucleotide differences. All haplotypes are found in the wild (Iwate population); H1 is absent from the captive population.

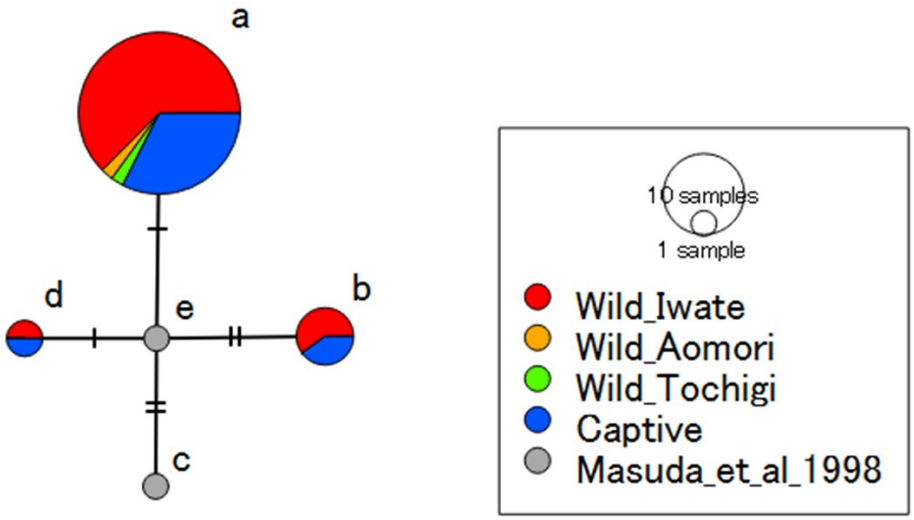


Figure A2.2 Median-joining network of ψCR haplotypes founded in Japan. It contains five haplotypes, however haplotype e and c was not founded from this study and referenced from Masuda et al. (1998). Three haplotypes were founded from 31 wild individuals and 16 captive individuals. Red colour indicates haplotypes observed in the wild Iwate-prefecture, orange in the wild Aomori-prefecture, green in the wild Tochigi-prefecture, and blue found in captive population. The circle size indicates the number of samples of each haplotype, and the number of dashes between each haplotypes means the number of nucleotide differences.

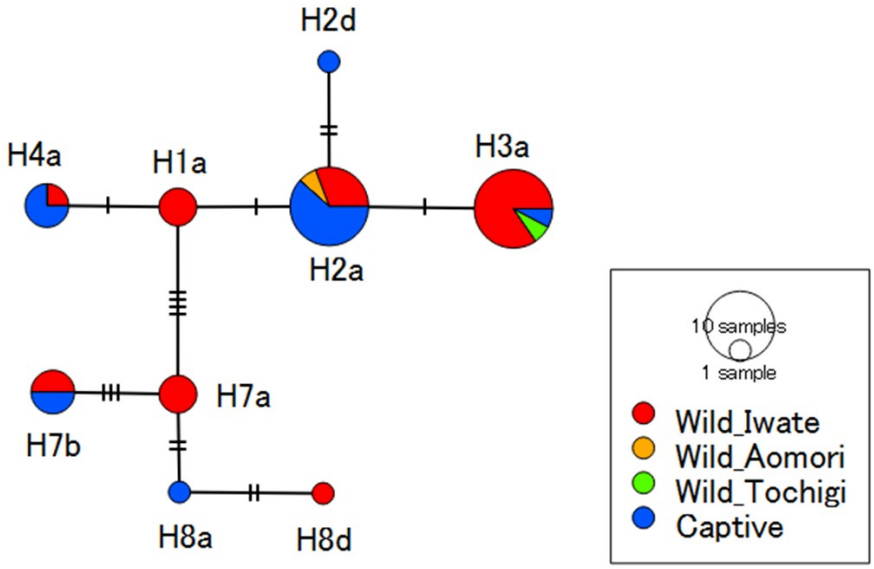


Figure A2.3 Median-joining network of concatenated CR + ψCR haplotypes found in Japan. It contains 9 haplotypes observed from 43 individuals. Circle size indicates the number of samples, and the number of dashes between haplotypes reflects the number of nucleotide differences. In addition to the sequence haplotypes described above, wild samples from one nest site showed additional novel haplotypes H18 and H19 (accession numbers: LC146690 and LC146691) in CR, and f (LC146689) in ψCR; however, in all cases these haplotypes were observed at sequence bases showing clear heteroplasmy (H18/H1, H19/H1, and a/f-type heteroplasmy) and were therefore not included in diversity calculations.

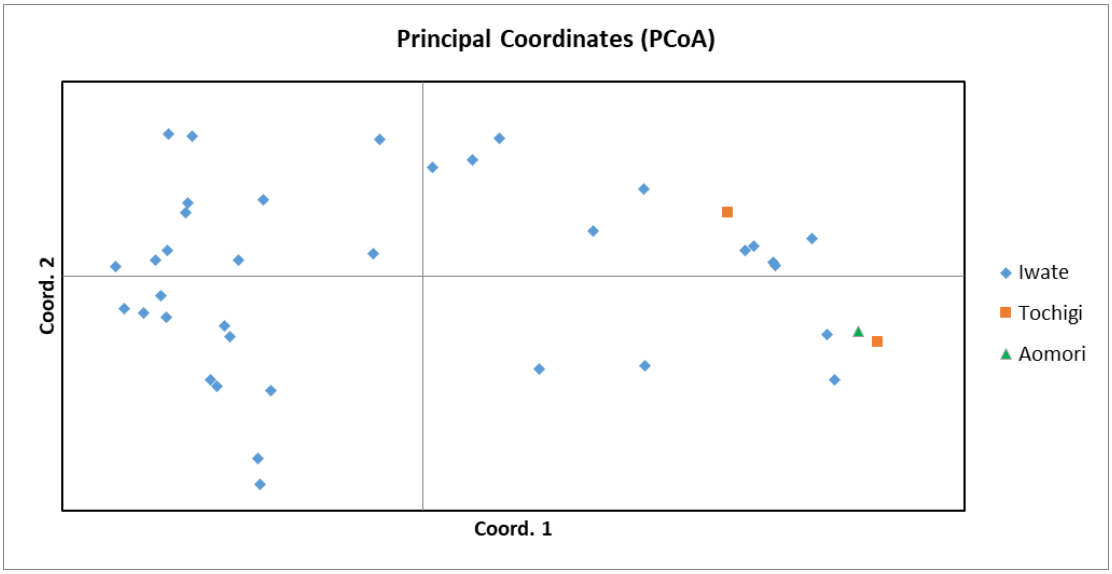


Figure A3 Result of PCoA analysis calculated from sixteen microsatellite loci of wild Japanese golden eagles. Thirty six samples were collected from Iwate-prefecture (blue), two samples were collected from Tochigi-prefecture (orange), and one sample was collected from Aomiri-prefecture (green). Tochigi is more than 400 km away from Iwate, however data from the first two principle coordinates do not show any geographic discrimination within among samples.

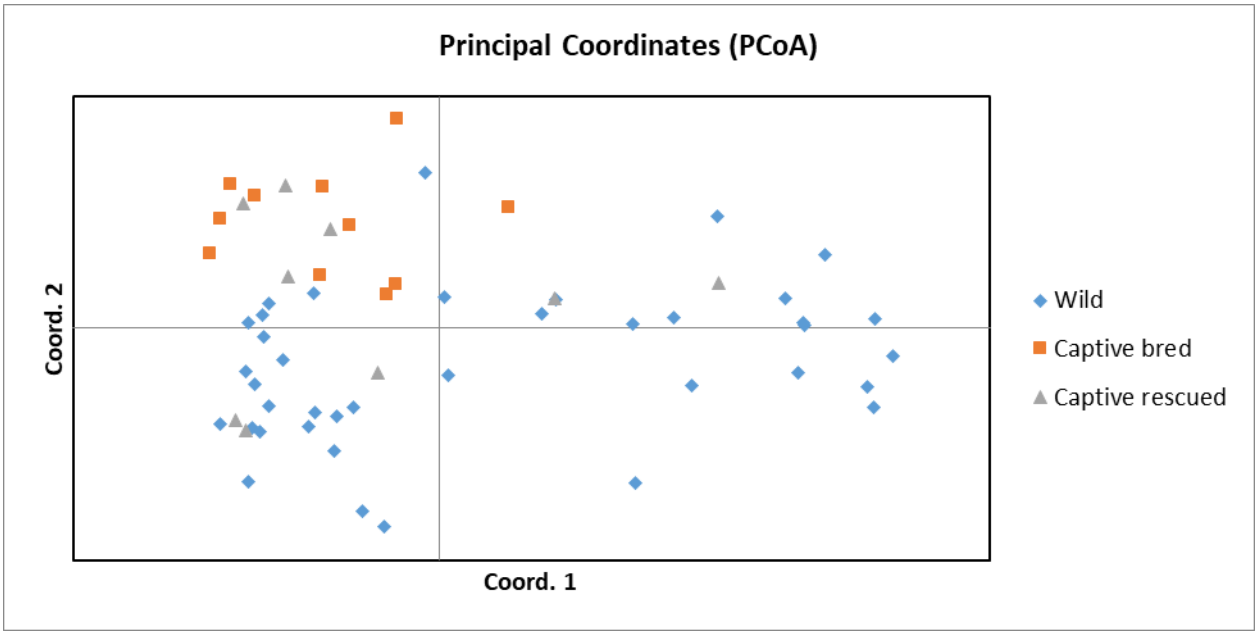


Figure A4 Result of PCoA analysis calculated from sixteen microsatellite loci of wild and captive Japanese golden eagles. Thirty nine samples were collected from the wild (blue), and twenty samples from captive birds (orange and gray). Wild samples were collected from Iwate (n= 36), Tochigi (n = 2), and Aomori (n= 1). Nine captive samples were from rescued individuals (Akita, n = 4, Miyagi, n = 1, Niigata, n = 2, Tokyo, n = 1, Fukui, n = 1, gray), and remaining samples were captive bred (orange).

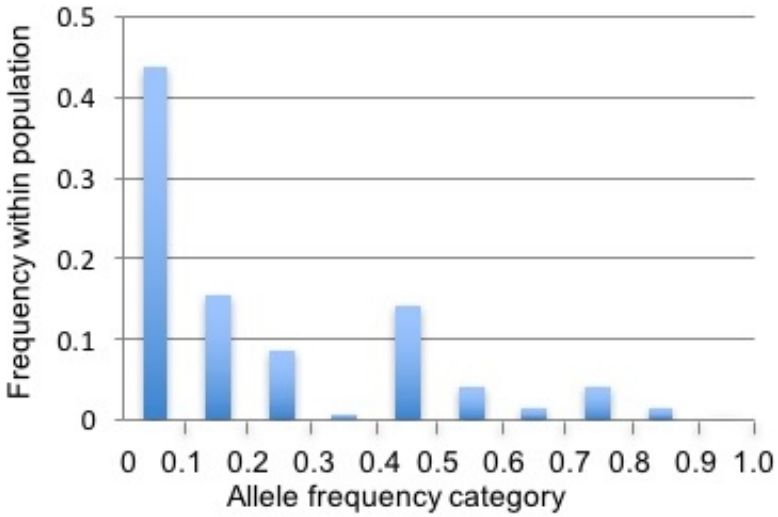


Figure A5 Graph to investigate mode-shift in allele frequencies as an indication of a recent genetic bottleneck in the Japanese wild golden eagle population. The observed L-shaped distribution does not provide evidence for a genetic bottleneck, despite observed demographic decline.